SEROLOGICAL EVIDENCE OF DENGUE INFECTION IN NON-HUMAN PRIMATES IN PENINSULAR MALAYSIA

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ABSTRAK

Jangkitan Virus Denggi (DENV) berlaku di kalangan manusia dan di dalam kitaran Silvatik. Pada tahun 2008 seorang pelajar universiti didiagnoskan dengan Demam Hemorajik Denggi (DHF) selepas ia mengembara di Semenanjung Malaysia dan pemencilan darah selanjutnya menunjukkan bahawa jangkitan itu adalah Serotip-2 DENV Silvatik. Kawasan Malaysia dengan kepadatan populasi yang tinggi didapati tidak mempunyai sebarang maklumat semasa berkenaan dengan kitaran DENV Silvatik. Oleh kerana DENV Silvatik menyebabkan penyakit teruk pada manusia, maka mengkenalpastikan lokasi dan spesies perumah pada kitaran jangkitan DENV Silvatik merupakan langkah kritikal yang pertama dalam penilaian risiko yang diutarakan oleh ejen zoonosis ke atas populasi manusia yang hidup berdampingan dengan spesies perumah ini. Tujuan utama kajian ini adalah untuk memperolehi antibodi peneutralan bagi DENV-2 dari beruk liar. Sampel darah telah diekstrak dari 430 *Macaca fascicularis*, 6 *M. nemestrina*, dan 6 *Semnopithecus cristatus* yang berhidup di lima negeri di Semenanjung Malaysia dan diperiksa dengan ujian Plaque Reduction Neutralization Test (PRNT). Antibodi peneutralan bagi DENV-2 telah ditemui pada 67 individu *M. fascicularis* dan 1 individu *M. nemestrina* yang disampel di Pahang, Negeri Sembilan, dan Selangor. Antara sampel-sampel yang didapati mengandungi antibodi

peneutralan bagi DENV-2, dua berasal dari kawasan dengan populasi Demam Hemorajik Denggi seramai 1.8 juta pesakit yang mana pemencilan darah selanjutnya menunjukkan bahawa ianya merupakan Serotip-2 DENV. Sampel peneutralan DENV-2 diperolehi dari 20 beruk dalam pensampelan tunggal di Negera Sembilan. Kekurangan bukti serologi dan ketiadaan pemencilan virus mencadangkan bahawa kitaran DENV Silvatik atau DENV manusia ditumpah kembali (spillback) kepada populasi beruk yang hidup berdampingan dengan populasi manusia yang berkepadatan tinggi. Kaedah kawalan denggi semasa tidak mengambilkira kesan-kesan limpahan zoonosis strain Silvatik ke atas populasi manusia atau kesan-kesan tumpahan kembali DENV manusia kepada spesies primate bukan-manusia walaupun kedua-dua kesan ini merupakan pertimbangan yang amat penting dalam kesihatan awam. Oleh yang demikian lebih banyak kajian lanjutan diperlukan untuk menentukan ancaman yang dihadapai oleh populasi manusia yang hidup di zon kemunculan ini.

SEROLOGICAL EVIDENCE OF DENGUE INFECTION IN NON-HUMAN PRIMATES IN PENINSULAR MALAYSIA

ABSTRACT

Dengue viruses (DENV) circulate in both human and sylvatic cycles. In 2008, a Malaysian university student was diagnosed with dengue hemorrhagic fever (DHF) and a sylvatic DENV serotype 2 was isolated from his blood. Current information on sylvatic DENV cycles in these heavily populated areas of Malaysia is nonexistent. Sylvatic DENV can cause severe disease (DHF) in humans. Therefore, the objective of this study was to determine the location and host species of sylvatic DENV cycles as a first step in assessing the risk that such zoonotic viruses pose to the surrounding human population. In this study, 430 *Macaca fascicularis*, six *Macaca nemestrina*, and six *Semnopithecus cristatus* monkeys from five states in Peninsular Malaysia were trapped from the wild and screened for DENV-2 neutralizing antibodies using the Plaque Neutralization Reduction Test (PRNT). DENV-2 neutralizing antibodies were found in 67 *M. fascicularis* (16%) and one *M. nemestrina* (17%) macaques from Pahang, Negeri Sembilan, and Selangor. Two seropositive monkeys were sampled from areas with a population of 1.8 million people. DENV-2 neutralizing samples were obtained from 20 macaques in a single outing in Negeri Sembilan. The limitations of serological evidence and absence of virus isolation suggest that the DENV-2 neutralizing antibodies detected in this study are evidence for either the presence of sylvatic DENV cycles or of human DENV spillback into macaques living near human populations. Current dengue control

measures do not account for the zoonotic spillover of sylvatic strains into the human population or the spillback of human DENV into non-human primates. The blood isolation of a DENV from a macaque is needed to establish the identity of the virus that currently circulates in primates living within these zones of emergence.

CHAPTER 1 – INTRODUCTION

1.1 Background

Dengue viruses (DENV) are the etiologic agents of two acute febrile diseases known as dengue (D) and severe dengue (SD) - a more severe presentation of the disease that includes severe hemorrhaging and organ impairment. (WHO 2009) This mosquito-borne viral disease is spreading rapidly with an enormous impact on public health: 2.5 billion people are exposed to the risk of dengue infection and the impact and scope of dengue epidemics worldwide has been on a precipitous rise, aided by unchecked human population growth in endemic regions and ensuing reclamation and repurposing of land. (WHO 2014) Given its pantropical distribution and the great number of people afflicted yearly, dengue merits consideration as a public health emergency of international concern.

Classified as arboviruses (athropod-borne viruses), dengue viruses belong to the genus *Flavivirus* and are known to occur in four antigenically distinct serotypes: DENV-1, 2, 3, 4 (Calisher *et al.,* 1989). Epidemics of dengue fever are primarily caused by strains of DENV that are restricted to humans as the principal reservoir and amplifying host. The main vectors of such infections are the domestic and peridomestic *Aedes aegypti* or *Aedes albopictus* mosquitoes. There also exist, in West Africa and Southeast Asia, separate and ecologically discrete strains of sylvatic enzootic dengue virus that circulate in non-human primates, utilizing forest canopy-dwelling Aedes spp. mosquitoes as vectors (Robin *et al.*, 1980). The epidemic (henceforth called endemic)

strains of DENV are thought to have emerged independently from sylvatic cycles (Holmes & Twiddy 2003) and viral spillover into human populations with severe disease has been documented in West Africa and, more recently, Peninsular Malaysia, South East Asia (Saluzzo *et al.,* 1986; Cardosa *et al.,* 2009; Vasilakis *et al.,* 2011). Sylvatic DENV has demonstrated the ability to spillover into a human population resulting in severe illness here in Malaysia, where a sylvatic DENV was isolated from the blood of a critically ill university student (Cardosa *et al.,* 2009) and West Africa (Franco *et al.,* 2011).

The mounting evidence that endemic DENVs diverged from their sylvatic ancestors relatively recently underscores the need to regard this zoonotic disease as a significant risk to public health. Consequently, assessing the potential of sylvatic dengue reemergence deserves attention of the highest priority. Globally, programs geared at reducing or eradicating dengue from human populations rely on vector control or the development of a future DENV vaccine. These efforts may prove less effective or even untenable should either one or both of two unknown scenarios prove true: 1) Endemic DENVs are able to be maintained by non-human primates where they can reemerge into a human population are the completion of a successful vector eradication/vaccination program or 2) Sylvatic DENVs spill over into and adapt to a human population through a bridge vector re-establishing an endemic transmission cycle after the completion of a vector eradication/vaccination program. The likelihood of both scenarios is currently still unknown.

1.2 Statement of the Problem

Studies conducted by Albert Rudnick in Selangor, Malaysia during the 1970s resulted in multiple isolations of sylvatic dengue viruses both from *Aedes* spp. vectors and non-human primates (Rudnick 1986). Over the past 30 years, large-scale development and repurposing of land in a dengue endemic country (Malaysia) has resulted in the concentration of human population in the zone of emergence, placing humans in direct contact with epizootic/sylvatic DENV cycles. A DENV virus isolated from a patient with severe dengue traveling in the same area yielded a DENV-2 that fell within the sylvatic clade and closely matched a sylvatic DENV-2 previously isolated by Rudnick (P8-1407/1970) (Cardosa *et al.,* 2009).

In spite of the rapid population movement and land development, there exists an information gap regarding the geographic location/distribution, prevalence, and strains of sylvatic DENV currently infecting forest/peri-domestic vectors and their primate hosts. Other unknowns include the ability of endemic dengue viruses to infect a sylvatic vector or sylvatic host, thereby reentering a forest cycle and rendering it protected from standard dengue virus control and eradication measures. If endemic dengue viruses originated from sylvatic lineages, understanding the circumstances in which sylvatic dengue spills into human populations is a question of paramount importance to public health.

Furthermore, there has been no research on these sylvatic DENV cycles in Malaysia in the past 30 years and much to uncover about how these enzootic cycles interact with endemic dengue and nearby human populations. To that end, ecological

studies which target specific areas most at risk for sylvatic dengue spillover into the human population should be conducted – for Southeast Asia, in the same general area of Selangor, Malaysia where the student who developed a sylvatic DENV infection traveled proximate to developing severe symptoms.

The aim of this study was to conduct a serosurvey of nonhuman primates for antibodies to DENV as a first step in determining if an enzootic sylvatic DENV cycle still exists in Peninsular Malaysia. Serum samples were collected from monkeys caught in traps or by routine wildlife officer patrols from Perhilitan / Department of Wildlife, Peninsular Malaysia from as many individuals and different locations as possible and screened for antibodies that bind DENV. The information that these ecological surveys provide will constitute a critical fist step in ascertaining the potential of a sylvatic dengue reemergence event and its potential threat to public health by identifying possible locations of enzootic DENV transmission.

CHAPTER 2 – LITERATURE REVIEW

2.1 Dengue Fever Clinical Classification

In 1997, the WHO classified symptomatic dengue virus infections into groups of three categories: undifferentiated fever, dengue fever (DF) and dengue hemorrhagic fever (DHF). DHF was further separated by severity into grade I, grade II, grade III and grade IV. Grade III and grade IV were defined as dengue shock syndrome (DSS). A rigorous literature review of this classification revealed difficulties in applying the criteria for DHF in a clinical situation (Bandyopadhyay, Lum and Kroeger 2006) resulted in a new set of WHO case classification guidelines in 2009.

Under the new classification system, symptomatic DENV infections are grouped into two main groups: dengue (D) and severe dengue (SD). D is further divided into D and dengue with warning signs (D + WS) (eg. abdominal pain, persistent vomiting, mucosal bleeding etc.) in order to better help clinicians with triage of symptomatic patients. (WHO 2009) While the D/SD was intended to replace DF/DHF/DSS, both classification systems are still in use as of the printing of this manuscript. In order to avoid the confusion that may result from converting from one classification system to another both systems are used here in accordance with the original publication referenced.

2.2 History and Spread of Dengue

Descriptions of fever and illness clinically compatible with the symptoms of dengue fever have been reported as early as 992 A.D. during the Northern Sung Dynasty in China. Epidemics of disease in Jakarta, Indonesia, Cairo, Egypt in 1780 may also have been dengue and it is thought that DENVs may have become endemic in these areas at this time (Gubler 1997). The first recorded outbreak/epidemic of dengue occurred in Manila, Philippines between 1953 and 1954. Dengue fever with hemorrhagic symptoms emerged in Bangkok, Thailand from 1962 to 1965 (Halstead 1965). In the proceeding 20 years, dengue epidemics began appearing in population centers throughout Southeast Asia leaving hospitalization and mortality in their wake (WHO 1986). During World War II, new advances in laboratory techniques led to the independent isolation of the first two serotypes of dengue virus, DENV-1 and DENV-2, by Japanese (Hotta 1952) and American (Sabin & Schlesinger 1945) researchers. The third and fourth serotypes, DENV-3 and DENV-4, were identified during an outbreak in the Philippines and Thailand in 1954 (Hammond, Rudnick and Sather 1960). By the 1980s and 1990s dengue had become a pandemic, spreading in cities from Southeast Asia, South Pacific, South Asia, to the Americas (WHO 2009)

2.3 Dengue Epidemiology

Dengue virus (DENV) belongs to the genus *Flavivirus*, of which other members include Japanese Encephalitis Virus (JEV), Yellow Fever Virus (YFV), and West Nile Virus (WNV). The DENV genome is a positive-sense, single strand of RNA

approximately 11,000 bases in length (Fields 2006). Infection with any one serotype of DENV leads to lifelong protection against the infecting serotype (homologous reinfection), however, only brief protection against another serotype (heterologous challenge) (Kurane & Ennis 1992). Human transmission of dengue is maintained by the domestic *Aedes aegypti* and peri-domestic *Aedes albopictus* mosquitoes (Hanley & Weaver 2008).

DENV is widely distributed and circulates in tropical and subtropical regions constituting a human health threat of disconcertingly large proportions with approximately 100 million infections per year resulting in case fatality rates from 0.2% in Paraguay and 0.63% in Malaysia all the way to 2.63% and 8.11% in Indonesia and Bhutan respectively (WHO 2014). 2.5 billion people are exposed to the risk of dengue infection and the impact and scope of dengue epidemics worldwide has been on a precipitous rise, aided by unchecked human population growth in endemic regions and the ensuing reclamation and repurposing of land. A recent study (Bhatt *et al.,* 2013) has attempted to more accurately assess global rates of dengue infection by combining cartographic approaches, longitudinal information and detailed dengue cohort studies with risk maps and known dengue occurrences worldwide: Bhatt *et* al estimate that there are 390 million dengue infections per year of which 96 million presented with clinical or subclinical severity – more than three times current estimates by the WHO. The emergence of dengue as a global pandemic has been linked to mass movement of troops during World War II, the growth of international travel, and rapid, unplanned urbanization in developing nations where dengue is endemic (Gubler 1998). As of 2011, most large urban centers in the tropics spanning the globe from Asia to the Americas are

hyperendemic with multiple serotypes of DENV circulating and causing disease (Gubler 2011).

2.4 Risk Factors for Development of Severe Disease

Determining the percentage of DENV infections that result in DHF of SD is difficult: Kurane & Ennis (1992) have described clinically apparent dengue fever as the tip of the iceberg in that less than 10% of symptomatic dengue cases are reported and more than 50% of dengue infections are asymptomatic (WHO 2014). Risk factors for SD/DHF include ethnicity, viral genotype, and sequence of infecting serotypes (Gubler 1998). Although the specific mechanism for DHF/SD is currently unknown, there are two competing hypotheses for the development of severe disease:

(i) The antibody (Ab)-dependent enhancement (ADE) theory posits that during a second, heterologous DENV infection, heterotypic antibodies from the previous infection bind DENV from the second infection but are unable to neutralize the virus. The Ab-virus complex then attaches to Fc receptors on circulating monocytes and macrophages that then internalize the infectious Ab-virus complex. Ab-virus complexes are more readily taken up than uncoated virus particles thereby increasing the efficiency with which monocytes and macrophages are infected by DENV resulting in higher levels of viremia and cytokine release in DHF patients (Halstead 1989; Kliks *et al.,* 1989; Whitehead *et al.*, 2007).

(ii) Certain phenotypes of dengue are more virulent than others – Rico-Hesse *et al.* (1997) compared nucleotide sequences in the E/NS1 gene region of the dengue virus genome to demonstrate the association of the introduction of two distinct DENV-2 genotypes and the appearance of DHF in the Americas.

2.5 Endemic Dengue Vectors

The principal vector for DENV is the anthropophilic, urban mosquito *Aedes aegypti*. Historically implicated by its correlation with the spread of dengue fever outbreaks, *A. aegypti* is now a fully domesticated mosquito and well adapted to its urban environment (Smith 1956; Powell 2013). Despite also being the primary vector for the Yellow Fever (genus: *Flavivirus*) and Chikungunya (genus: *Alphavirus*), its importance in Asia is primarily in relation to DENV and not Yellow Fever due to the high morbidity and mortality of the former and the absence of the latter in Asia (Barrett & Higgs 2007).

The peridomestic/forest fringe mosquito *Aedes albopictus* is also DENV competent and Smith (1956) noted its presence in rural areas where human Abs to dengue were present, *A. aegypti* was absent. In a household vector survey in Dhaka, Bangladesh during a DF and DHF outbreak Ali *et al.* (2003) found that households reporting a recent dengue illness were more likely to have *A. albopictus* larvae present within the home compared to households not reporting cases implicating *A. albopictus* as the sole vector in some cases.

2.6 Enzootic / Sylvatic Dengue Cycle

Sylvatic Dengue in West Africa

In contrast with Asian sylvatic dengue, a greater number of studies have canvased the ecology of sylvatic DENV transmission in West Africa and, broadly, as of 2015, the West African cycle is characterized by the presence of DENV-2 as the sole strain and an approximately eight-year cycle (Robin *et al.*, 1980; Cornet *et al.*, 1984; Saluzzo *et al.*, 1986; Zeller *et al.*, 1990; Cornet 1993, Diallo *et al.,* 2003) The first conclusive evidence for an enzootic DENV cycle in West Africa was a the isolation of a sylvatic DENV-2 virus from a human near the Senegalese capital Dakar in 1970 and a sylvatic DENV-2 from forest-caught pools of *Aedes luteocephalus* near the town of Kédougou in southeastern Senegal (Robin *et al.*, 1980). Serosurveys, in search of a forest dwelling host, conducted in Senegal from 1974 and 1985 gathered proof that forest primates served as a reservoir (Saluzzo *et al.*, 1986). In one study, neutralization assays in Nigeria found DENV neutralizing antibody in monkeys (48%) and galagos (25%). Additionally, the same study found that 48% of humans with DENV neutralizing antibody were living in "Rainforest Zones" far removed from endemic DENV vectors (Fagbami, Monath and Fabiyi 1977). Human endemic DENVs were isolated from forest mosquitoes *Aedes furcifer*, *A. taylori*, and *A. luteocephalus* casting suspicion on these mosquitoes as putative bridge vectors in forest dengue cycle as these mosquitoes lived in forest and forest fringe settings unlike the urban *A. aegypti* and peridomestic *A. albopictus* species (Diallo *et al.*, 2003). The recovery of human, endemic DENV from these mosquitoes implies that they had previously fed on humans.

While sylvatic DENV strains are considered to have been the phylogenetic progenitors of DENVs transmitted among humans, none have yet been identified as the etiologic agents of dengue outbreaks in human populations. With the hypothesis that sylvatic DENVs may cause undetected spillover epidemics, a team from the University of Texas Medical Branch, examined isolates of DENV recovered from febrile patients at University College Hospital in Ibadan, Nigeria from August 1964 to December 1968. After amplification with RT-PCR, sequencing, and phylogenetic analysis, 3 of 32 original Nigerian isolates, all three from 1966, were reported as genetically distinct from endemic DENV-2 isolates and fell within the sylvatic DENV-2 clade – evidence suggesting that that a sylvatic DENV cycle had spilled over into humans in Ibadan, Nigeria in 1966 (Vasilakis, Tesh and Weaver 2008). Virus isolations of sylvatic DENV-2 continued to lend epidemiological support to the existence of enzootic cycles in West Africa, until a sylvatic DENV-2 strain was isolated from *Erythrocebus patas* monkeys in Eastern Senegal (Cornet 1984). From 1999 to 2000, 6 sylvatic DENV-2 strains were isolated from forest mosquitoes (*A. furcifer*) collected from inside a the town of Kedougou in Southeastern Senegal (Diallo *et al.,* 2003). Human endemic DENVs were also recovered from forest mosquitoes *Aedes furcifer*, *A. taylori*, and *A. luteocephalus* mosquitoes living in forest and forest fringe (Diallo *et al.*, 2003). Given the absence of urban *A. Aegypti* and peridomestic *A. Albopictus* species in a rainforest environments, it is possible that *A. furcifer*, *A. taylori*, and *A. luteocephalus* may serve as potential bridge vector between sylvatic DENV transmission cycles in non-human primates and nearby human populations. More importantly, while sylvatic DENV has been demonstrated in mosquitoes, monkeys, and (recently) humans, studies conducted thus far have not isolated/identified sylvatic strains to be active in vector, reservoir, and humans

concurrently in space and time, therefore limiting the epidemiologic information that can be gleaned about the conditions under which viral spillovers occur in West Africa.

Sylvatic DENV is capable of causing severe illness in humans: In November 2009, a 27 year-old man returning to Spain from his home country of Guinea-Bissau, by way of Senegal, in West Africa was admitted to hospital with symptoms consistent with Grade II Dengue Hemorrhagic Fever. Serology, PCR, and phylogenetic analysis of the causative agent revealed something unexpected: A sylvatic DENV of West African lineage designated EEB-7 (Franco *et al.,* 2011).

Sylvatic Dengue in East Asia

Unlike West Africa, DENV-1, 2, 4 have been detected in Asia, suggesting the prospect of hyperendemicity and the near constant circulation of at least one of the three serotypes. The first work to lend solid support to the existence of sylvatic DENV was that of Gordon Smith on the west Malaysian peninsula during the 1950s. Serological studies conducted on rural Malay communities revealed that 80% of adults possessed dengue neutralizing antibodies in areas where *A. aegypti* mosquitoes, the urban vector for endemic DENV, were completely absent (Smith 1958). Humans were not the only species testing positive – Smith (1956) found large numbers of aroboreal mammals with neutralizing antibodies such as civets, squirrels and slow lorises (*Nycticebus coucang*, a non-human primate). He speculated that *A. albopictus* might maintain dengue endemicity in rural settings due to its very large numbers (Smith 1956; Smith 1958). Smith hypothesized that the presence of DENV neutralizing antibodies in humans and

primates far removed from endemic DENV transmission foci and its vector implied the existence of a separate, enzootic DENV cycle in rural and forested areas. The existence of the cycle, however, remained unsubstantiated due to a lack of DENV isolation from any potential forest vectors.

By far, the most extensive and intensive DENV ecology surveys ever performed in Asia are those of Albert Rudnick performed a decade after Smith during his association with Malaysia's Institute for Medical Research from 1962 to 1980. Rudnick examined forest mammals and primates in habitats ranging from primary and peat swamp forests to heavily disturbed plantation areas united by the absence of *A. aegypti*. Serology revealed the presence of DENV neutralizing antibodies in more than 47% of *Macaca nemestrina, Macaca fascicularis* (68%) and *Trachypithecus cristatus* (Rudnick 1965) corroborating Smith's (1956) earlier conclusions of DENV as a zoonosis. Spurred by his initial findings, Rudnick stationed *Maccaca* and *Trachypithecus* monkeys as sentinels and live bait for mosquitoes in the forest canopy. He succeeded in isolating DENV-1 (strain P72-1244), three strains of DENV-2 (P8-1407, P72-1273 and P72-1274), and three strains of DENV-4 (P75-481, P73-1120 and P75-514) from sentinel macaque sera. Rudnick did not recover any isolates from any of 19 ground-stationed *M. nemestrina* sentinels which may indicate that transmission took place in jungle canopies (Rudnick 1986). Concurrent mosquito collections yielded a DENV-4 (P75-215) isolate from the sylvatic, canopy dwelling *Aedes niveus.* Rudnick noted that he increased capture numbers of *A. niveus* considerably when the using human bait instead of dry ice or caged macaques (Rudnick 1986) demonstrating that *A. niveus* opportunistically fed on humans. The most recent search for neutralizing antibodies in non-human primates was

a serosurvey conducted in Borneo by Wolfe *et al.* (2001) found DENV-2 specific antibodies in orangutans. And yet these primates are unlikely to be a sylvatic dengue reservoir due to their extremely low population density of 1 to 2 orangutans per km^2 – it would be unlikely for an infected mosquito to travel the kilometers between blood meals necessary to maintain transmission.

The role of the common, peri-domestic *A. albopictus* as a possible vector of sylvatic dengue is a subject of contention. Simmons et. al in 1931 demonstrated experimentally that *A. albopictus* is an efficient endemic DENV vector. This mosquito species was later implicated in a dengue fever outbreak in port cities of Japan during WWII (Kimura & Hotta 1944; Hotta 1952) and is also a sylvatic DENV vector suspect due to its often large numbers near human habitation in forest fringe settings and rural land converted to palm oil plantations (Chang *et al*., 1997). Although *A. albopictus* was already known as a competent endemic dengue vector, Moncayo *et al.* (2004) compared the infection and dissemination rates of endemic and sylvatic DENV-2 in both *A. aegypti* and *A. albopictus* to determine if endemic dengue had to adapt to these peridomestic mosquitoes as a step before cementing the ability to infect humans on a large scale. They discovered that while both *Aedes* species were highly susceptible to endemic dengue infection, they were significantly less susceptible to sylvatic DENV-2. The Southeast Asian DENV-2 isolated by Rudnick in 1970 had some of the lowest infection rates and the lowest dissemination rate (0%). Furthermore, Rudnick's isolation of DENV-2 from an *A. albopictus* mosquito in 1969 was, in retrospect, an *endemic* strain of DENV-2 according to phylogenetic analysis (Wang *et al*., 2000). While *A. albopictus* still remains a likely candidate for a sylvatic dengue bridge vector, these studies

collectively suggest that other *Aedes* mosquitoes are also competent and merit further study. It is also possible that normally primatophillic mosquitoes shift their feeding preference from forest primates to humans. A change in mosquito feeding behavior has been implicated in the spread of West Nile Virus (also a *flavivirus* like DENV) in the U.S.A. (Gómez *et al.,* 2008). Given Rudnick's observation that *A. niveus* mosquitoes displayed a preference for biting humans when given the choice between carbon dioxide bait, macaques and humans, a change in forest-dwelling *Aedes* mosquito feeding behavior may play a role in sylvatic DENV spillover event. Hypothetically, an *Aedes* mosquito normally involved in an enzootic DENV transmission cycle opportunistically shifts its feeding preference to humans allowing the transfer of sylvatic DENV into the human host/population. As more time passes, the sylvatic DENV competent mosquito may shift its preference to humans entirely resulting in the emergence of sylvatic DENV in a human population.

In January of 2008, a 20-year-old male student at Universiti Malaysia Sarawak was admitted to hospital after developing DHF Grade II two days after he had returned home from a four-week stay in Peninsular Malaysia (where he traveled along the western slopes of the Main Range). Blood drawn on day four of his fever revealed the causative agent to be a DENV-2. A DENV-2 isolate (DKD811) was cultured from blood samples and sequenced. Subsequent phylogenetic analysis yielded unexpected results: DKD811 was a sylvatic DENV-2 strain (Cardosa *et al.*, 2009). Not only was this the first recorded case of DHF in a human of sylvatic DENV origin, but it was also the first definitive case of a human acquiring sylvatic DENV in Malaysia. Further phylogenetic investigation and molecular clock analysis closely matched DKD811 with

a sylvatic DENV-2 strain (P8-1407/1970) isolated from a monkey in Tanjong Rabok, Selangor, Malaysia by Rudnick in 1970, making the new isolate the first evidence of sylvatic dengue in Asia since the 1970s (Cardosa *et al.*, 2009). The sylvatic strain P8-1407 is closely matched with DKD811 and was isolated from this *same* general location (Rudnick 1986). At the conclusion of his studies in peninsular Malaysia, Rudnick (1986) had successfully isolated sylvatic strains of DENV-1, DENV-2, and DENV-4 from *Aedes* species mosquitoes or non-human primates and although a sylvatic DENV-3 was never isolated, its existence was inferred from Rudnick's detection of DENV-3 neutralizing Abs in non-human primates.

Recently, Teoh *et al.* (2010) retrospectively analyzed the envelope gene sequqnce of 442 endemic DENV-1 isolates from a 2004 - 2007 dengue fever outbreak in Malaysia. One isolate (D1.Malaysia.36046/05) shared 97% nucleotide sequence and 99% amino acid sequence similarity with isolate P72-1244 – a DENV-1 isolated by Rudnick (1986). Hospital records indicated that the virus was isolated from a patient that displaying symptoms consistent with dengue at University of Malaya Medical Centre (Teoh 2010). Although D1.Malaysia.36046/05 was originally isolated from a sentinel monkey (*M. fascicularis*) in a rural area of peninsular Malaysia in 1972, it's status as a sylvatic strain has come in to question because it has recently been determined to be phylogenetically more similar to endemic DENV-1 (Rudnick 1986; Vasilakis and Weaver 2008). The detection of the isolate is important in either outcome: If sylvatic, it would be the latest example of a dengue zoonosis into a human host. If originally endemic, it demonstrates that endemic DENVs can spillback into monkeys.

Sylvatic Dengue Elsewhere

Evidence for the presence of a sylvatic DENV cycle in the Americas comes from serovconversion (by PRNT) to DENV-2 of Native Americans living in the remote area of Ayoreo, Bolivia (Roberts *et al.,* 1984). RNA representing four DENV serotypes was detected in marsupials, rodents, bats and deer from liver and/or sera – the first evidence of dengue virus infection of neotropical forest mammals (de Thoisy *et al.,* 2009). According to de Thoisy *et al.* (2009), "Sequence analyses of a portion of the capsid and premembrane junction revealed that mammal strains of DENV-1, DENV-2, DENV-3, and DENV-4 had only 92.6%, 89%, 95%, and 95.8% identity, respectively, with strains circulating in the human population during the same periods." Although the short RNA sequence evidence suggests that these DENV detections may be sylvatic, only complete virus isolation and complete genetic characterization can definitively distinguish between an endemic and sylvatic DENV. Another possibility is that these detections in French Guiana may be endemic DENVs that have spilled back into forest mammals.

2.7 Origins of Dengue

Zanotto *et al.* (1996) reconstructed the phylogeny of 123 complete DENV envelope genes in a maximum likelihood tree for each of the four serotypes to determine how the four endemic serotypes were related to each other. According to their analyses, DENV-4 was first to diverge followed by DENV-2 then DENV-1 and DENV-3. The origin of dengue still remained uncertain. Had it been with humanity from the dawn of our species or had it emerged relatively recently? Gubler (1997) posited that endemic

DENV originated from a sylvatic form of the virus that circulated in non-human primates and forest *Aedes* mosquitoes as vectors. Wang *et al.* (2000) tested Rudnick's hypothesis by sequencing the complete envelope (E) protein gene of DENV-1, -2, and -4 strains collected by Rudnick (1986) during his forest surveys in Malaya during the 1970s as well as sylvatic DENV-2 strains from West Africa. Notably, Wang *et al.* (2000)'s phylogenetic trees placed the African and East Asian sylvatic isolates at positions that were basal to their respective dengue serotype groups:

"[It is] estimated that the sylvatic DENV-2 genotypes diverged from the endemic/epidemic forms on the order of 1,000 ± 500 years ago (estimate ± standard deviation). The African and Malaysian sylvatic lineages of DENV-2 diverged on the order of 800 ± 400 years ago. Sylvatic and endemic/epidemic DENV-1 and -4 probably diverged on the order of 200 ± 100 and 600 ± 300 years ago respectively."

These estimates for DENV emergence also coincide approximately with the rise of human population centers large enough to support epidemic transmission of dengue (Gubler 1997, Wang *et al.,* 2000). The asynchronous divergence estimates yielded by phylogenetic analyses suggest the endemic/epidemic lineages of DENV-1, -2, and -4 evolved/emerged independently of one another from sylvatic viruses circulating in the Asian-Oceania region (Wang *et al.* 2000) – in other words, these emergence events happened separately *and* repeatedly for each dengue serotype giving rise to the four known endemic serotypes in circulation today. Gubler (1997) hypothesized that each of

these emergence events would have involved a sylvatic DENV switching from forest canopy-dwelling *Aedes* mosquito vectors to *A. albopictus* and later *A. aegypti.* Since this would have presumably happened in rural locations lacking *A. aegypti, A. albopictus* or other *Aedes* mosquitoes may have been the original human vectors (Smith 1956).

Twiddy, Holmes and Rambaut (2003) conducted a study similar to Wang *et al.* (2000)'s phylogenetic study of sylvatic and endemic DENV envelope (E) gene sequences, but incorporated additional information about times of virus sampling. Although in general agreement with Wang *et al.* (2000) that dengue virus appeared circa 1100 years ago, Twiddy *et al.* (2003) estimate that the zoonotic divergence events (the transfer of virus from monkeys to humans) of endemic from sylvatic dengue are nearly three times more recent:

"The dates for the data sets that combine human and sylvatic strains are approximately 320 years ago for DENV-2, 200 years ago for DENV-4, and 125 years ago for DENV-1 . . . For all four serotypes, these estimates place the beginning of the epidemic transmission of dengue in humans near to the end of the nineteenth and the beginning of the twentieth century."

Hanley and Weaver (2010) theorize that if Twiddy *et al.*'s estimates are correct, then DENV strains that caused epidemics before the 18th century may have gone extinct and been replaced by more recent sylvatic strains.

The question as to the origin of the four extant serotypes of DENV is still open today. Presumably, it is unlikely that the serotypes would diverge from the same/shared geographic location due to robust and enduring host immunity to homologous reinfection – any virus wasn't significantly different antigenically would be competitively excluded. Rather, it is more likely that the four serotypes of DENV are allopatric and evolved from sylvatic strains that were geographically isolated relative to one another. Once human population centers had become large enough, these allopatric sylvatic serotypes then emerged in separate events evolving into the four known endemic serotypes (Vasilakis *et al.,* 2007). Once the four serotypes had significantly diverged so as to offer only limited cross-reactive immunity upon heterologous challenge, the rise of international travel/commerce and concomitant spread of *Aedes aegypti* facilitated the spread of multiple serotypes resulting in areas of hyperendemicity in the tropics (eg. Malaysia) (Gubler 1997, Vasilakis *et al.,* 2007). If all four serotypes of DENV emerged recently and *independently* as Twiddy *et al.* (2003) estimate, then the probability of a sylvatic DENV strain spilling over into a human population and evolving to a primarily human host/vector cycle is larger than if only one such even had taken place in the past.

2.8 Possibility of Spillover or Sylvatic Dengue Emergence

While studying the Yellow Fever Virus (genus *Flavivirus*) in the Central African Republic, Germain (1976) noted the high density of peridomestic and forest *Aedes* vectors in the boundary between the forest and savanna ecotopes where many isolates of

Yellow Fever had previously been recovered from humans and mosquitoes and where humans and forest animals were both present. Germain (1976) coined the term "zones of emergence" to refer to such places where sylvatic cycles of disease could spillover and cause human pathology. As global population increases and forest is cleared for development these zones of emergence are growing larger and more common.

This is especially true of developing countries in the tropics. Malaysia is a prime candidate that fits many risk criteria for emergence: (i) it is developing rapidly with resultant deforestation and changing land use creating "zones of emergence" (ii) sylvatic DENV-1, DENV-2, and DENV-4 have been isolated from non-human primates and forest/peridomestic *Aedes* vectors (Rudnick 1986) (iii) a sylvatic dengue virus has caused severe disease (DHF) in humans (Cardosa *et al.,* 2009) (iv) and endemic dengue viruses may be spilling back into forest and forest fringe monkeys (Teoh 2010).

Some are skeptical if sylvatic dengue viruses pose a threat to public health. In a reviewing the role of monkeys in the biology of dengue, Rodhain (1991) states:

"The constant reduction in size of natural forests tends to make the original simian epidemiologic cycle somewhat of a relic which, at present, has practically no importance as a reservoir. It would be necessary to take it into consideration only if, before its disappearance the inter-human died out, for example as the result of vaccination . . ."

While it may be true that enzootic DENV hosts like *M. fascicularis* are on the decline, they also show remarkable ability to adapt and thrive in urban peri-urban environments and it remains to be seen if they will eventually adapt entirely to an urban environment as in the case of 131 *M. fascicularis* recently discovered living in the middle of Bangkok, Thailand (Eudey 2008; Chatpiyaphat and Boonratana 2013). While habitat destruction continues, sizeable forest reserves in the form of national parks (eg. Taman Negara in Malaysia) may be large enough to maintain sylvatic transmission cycles. Whatever the truth of the matter may be, one fact remains: No data, serological or otherwise, has been collected about the whereabouts of sylvatic transmission cycles in non-human primates in Malaysia for almost four decades (Rudnick 1986) and any hypotheses regarding the presence, decline, or absence of these sylvatic DENV cycles can be supported or refuted without new/current data.

CHAPTER 3 – MATERIALS AND METHODS

3.1 Primate Serum Samples

Serum samples were received from the Department of Wildlife and National Parks (Perhilitan), Peninsular Malaysia headquarters in Cheras, Selangor in aliquots ranging from 50 – 60 µl in 1.5 ml Eppendorf microfuge tubes. Officers of the ex-situ team, DWNP, obtained these blood samples during outings including, but not limited to DWNP surveys and nuisance animal relocations. These samples were collected by DWNP staff during officially sanctioned, routine outings for the department's own use during 2009. No sample collection was specifically performed for this study – As such, no ethics release was required. Samples were transferred from freezer storage at -20 ºC at Perhilitan into thick-walled polystyrene coolers packed with dry ice −78.5 °C then immediately transported by car to the Venture Technologies Sdn Bhd laboratory at the Institute for Research in Molecular Medicnine (INFORMM), Universiti Sains Malaysia, Pulau Pinang where they were stored at −80 °C. Sample processing flow is summarized in Figure 3.1.

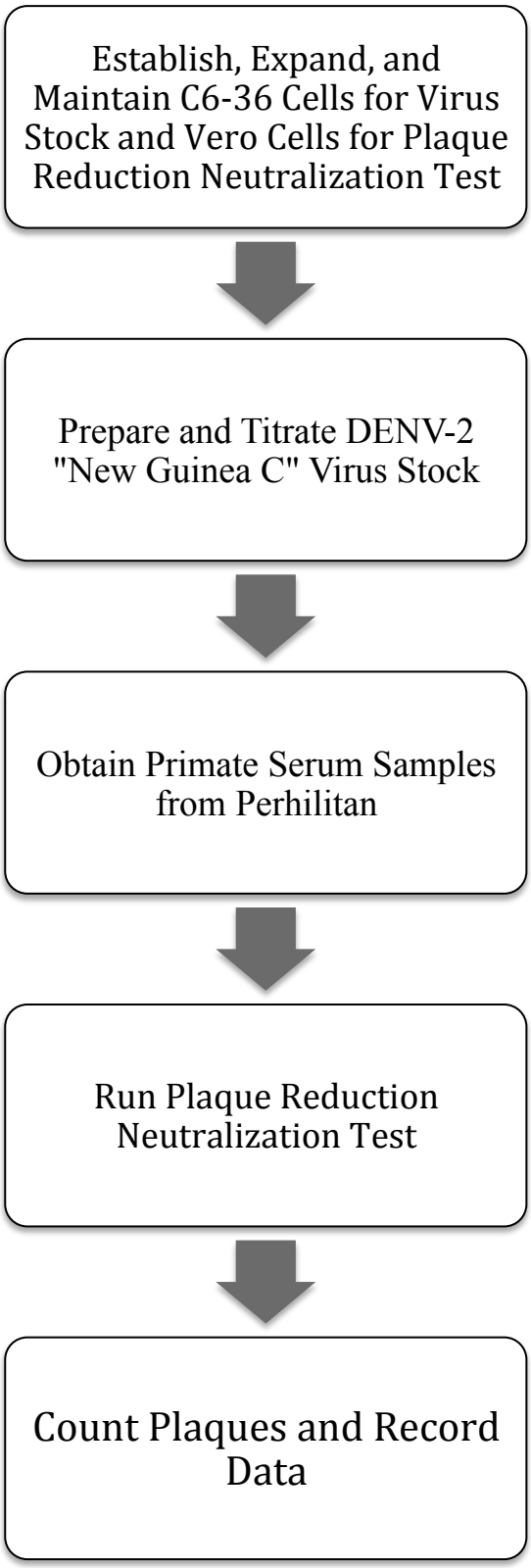

Figure 3.1 Flowchart of research acitivities.